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In vitro Study on Gametophyte Development of an Epiphytic Fern, *Arthromeris himalayensis* (Hook.) Ching, of South Sikkim, India

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ABSTRACT.—Gametophyte development in *Arthromeris himalayensis* was studied and found to be of "*Drynaria* type". Germination occurred 9–10 days after sowing of spores. Some prothalli showed an initial archegonial phase, which persisted throughout gametophyte development and the antheridial phase developed on separate thalli a few days later and persisted throughout the life span of the gametophytes. This type of development of sex organs may be considered as a variant new type from the previously reported types by earlier authors. This variant type is described here as "type H". This type of gametangial development on separate prothalli is an indication of adaptation for out breeding.

KEY WORDS.—Gametophyte development, *Arthromeris himalayensis*, Epiphyte, Type H, out-breeding

Arthromeris himalayensis (Hook.) Ching belongs to the family Polypodiaceae and is a warm temperate fern, exclusively epiphytic in nature and distributed in India throughout the Himalayan region from Eastern Himalayas to Western Himalayas. This epiphytic fern is also found in China, Nepal and Burma in mountain areas. In Southern Sikkim this fern is generally found between 2700–3600 m.

Germination of fern spores, growth, and further development of resulting gametophytes in artificial media is a well-studied area in pteridophyte and developmental biological research (Nayar, 1962; Atkinson and Stokey, 1964; Kato, 1969; Klekowski, 1969; Nayar and Kaur, 1969; Nayar and Kaur, 1971; Masuyama, 1975a, b; Khare and Kaur, 1983; Raghavan, 1989; Chiou and Farrar, 1997; Verma *et al.*, 2000; Verma, 2003; Ganguly and Mukhopadhyay, 2005). Nayar and Kaur (1971) and Atkinson (1973) pointed out that the sequence and plane of cell divisions, pattern of gametophyte development, as well as the direction of initial growth of the first rhizoid and germ filament with respect to the polarity of germinating spore are distinct characteristics and can be utilized effectively for drawing phylogenetic relationships among various taxonomic groups. Nayar (1962) studied the spore germination and prothallial morphology of *Arthromeris wallichiana* (Sprengel) Ching along with some other polypodiaceous ferns. However, no work has been done on the prothallial development of the epiphytic fern *Arthromeris himalayensis*. Ferns

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occupy a specialized habitat as epiphytes and, as such, epiphytic ferns have evolved various gametophytic generation adapations like antheridiogen systems, production of gemmae, indefinite growth of prothalli, etc. (Farrar, 1974, 2003). Among the 15 species of *Arthromeris* distributed throughout the world, the majority (10 species) are found in the Eastern Himalayas (Ghosh *et al.*, 2004). Out of these 10 species, *Arthromeris himalayensis* grows very successfully in the highest altitudes as epiphytes and has high antimicrobial activity (Ganguly *et al.*, 2008). These interesting attributes prompted us to study its reproductive behavior and gametophyte development. The current study was performed to understand the details of gametophyte structure and development pattern of prothalli in *Arthromeris himalayensis*.

Materials and Methods

Mature sporophylls of *Arthromeris himalayensis* were collected from healthy plants from Maenum Wildlife Sanctuary (3023 m altitude), South Sikkim in the November of 2005 and 2006. Sporophylls of individual plants were kept separately within blotting paper and mature spores from sporophyll(s) of individual plants dehisced within 48 hrs were collected in separate vials for gametophyte studies. Collected spores from three individual sporophytes were sown in separate petri plates on modified Moore's medium (Kato, 1969). Two replicates of each set were maintained. These spores were surface sterilized by 0.1% $HgCl_2$ (w/v) solution for 5–8 minutes and rinsed three times with sterilized distilled water and then dried on sterilized blotting paper. The sterilized spores were transferred to autoclaved (at 15 lb/inch^2 for 15 minutes) modified Moore's culture medium (Kato, 1969) solidified by 1% (w/v) agar in an aseptic chamber and the pH of the medium was maintained at 5.8. The cultures were incubated at 22°C–25°C under cool fluorescent white light (ca 1000 lux, 16hr/d).

Gametophytes from each petri plate were studied every day randomly by light microscope (Leica DMLB) after germination of spores. Time taken for spore germination and to form mature gametophytes, initiation of sex organs and formation of sporophytes were recorded. Camera Lucida drawings of different developmental stages were made on the same microscope. Records of gametophyte development patterns from individual sporophytes were maintained separately in order to see if there were variations in the developmental patterns among the individuals. Observations were made on ten gametophytes from each petri plate at a time.

Results

Characteristics of spore germination and gametophyte development.— Spores were bilateral, monolete; light brown in color, perisporate, perispore thin, size 38–42 × 50–53 μm. Spore germination of *Arthromeris himalayensis* was 79.49 ± 4.66%. Spores germinated even after having been stored for two months after collection.

Spores germinated 9–10 days after sowing. In this species the rhizoidal cell formed first (Fig. 1A) followed by the chlorophyllous protonemal cell (Fig. 1B). The protonemal cell developed into a 6-celled stage by periclinal divisions (Fig. 1C–E). Spore germination resulted in a slender uniseriate germ filament. The penultimate protonemal cell underwent oblique vertical division. In *Arthromeris himalayensis* the establishment of an apical meristem was much delayed and the prothalli usually developed hairs on the margin and surfaces. A broad spathulate prothalli plate was formed by repeated longitudinal and transverse divisions of its anterior cells and expansion of the resultant daughter cells (Fig. 1F–K). Mature vegetative, cordate shaped gametophytes (Fig. 1M) developed 77–80 days after spore germination. The mature prothalli measured ca 350 × 300 μm in size. The prothallial plate often became 15–20 cells or more wide and broadly ovate, but was devoid of any organized meristem. Later, an obconical meristematic cell was differentiated by two oblique divisions in one of the marginal cells at the anterior end of the prothallial plate (Fig. 1K). The meristematic region (Fig. 1L–M) was located under notch. The type of development was purely "*Drynaria* type" as discussed by Nayar and Kaur (1969, 1971).

Development of sex organs (sequence, position and duration).—Mature cordate gametophytes remained vegetative for about 30 days, after which the gametophytes started to develop sex organs. Archegonia developed first in some cordate shaped prothalli 112 ± 2 days after spore germination. Archegonia were situated along the midrib region and just below the meristematic region. Archegonia consisted of a projecting neck (Fig. 1M) and a lower embedded venter. This flask shaped structure was made up of two axial rows of neck canal cells, one ventral canal cell and one egg cell (Fig. 1N). Each archegonium had a single layered jacket (Fig. 1N) and was 150–200 × 65–75μm in size. Antheridia developed on separate gametophytic prothalli, which were elongated and much longer than archegonial prothalli. Initiation of antheridia started 115 ± 2 days after spore germination. Antheridia were of the emergent type (Fig. 1O) with a 1-cell thick jacket, measuring about 25–30μm.

In *Arthromeris himalayensis*, the prothalli were dioecious. The cordate shaped prothalli developed archegonia after they reached maturity and remained as archegoniate prothalli throughout the reproductive phase. Antheridiate prothalli were elongated and did not form well-defined apical meristem. Antheridia developed on the lower half of the prothallus, marginal and/or superficial in position (Fig. 1L). After initiation, antheridia took about 3–5 days to mature; spermatozoids were released after this period. The time taken for development of the different gametophytic stages of *Arthromeris himalayensis* is shown in Table 1.

Discussion

From the above observations, we can conclude that the type of gametophyte development in *Arthromeris himalayensis* is purely "*Drynaria* type". In

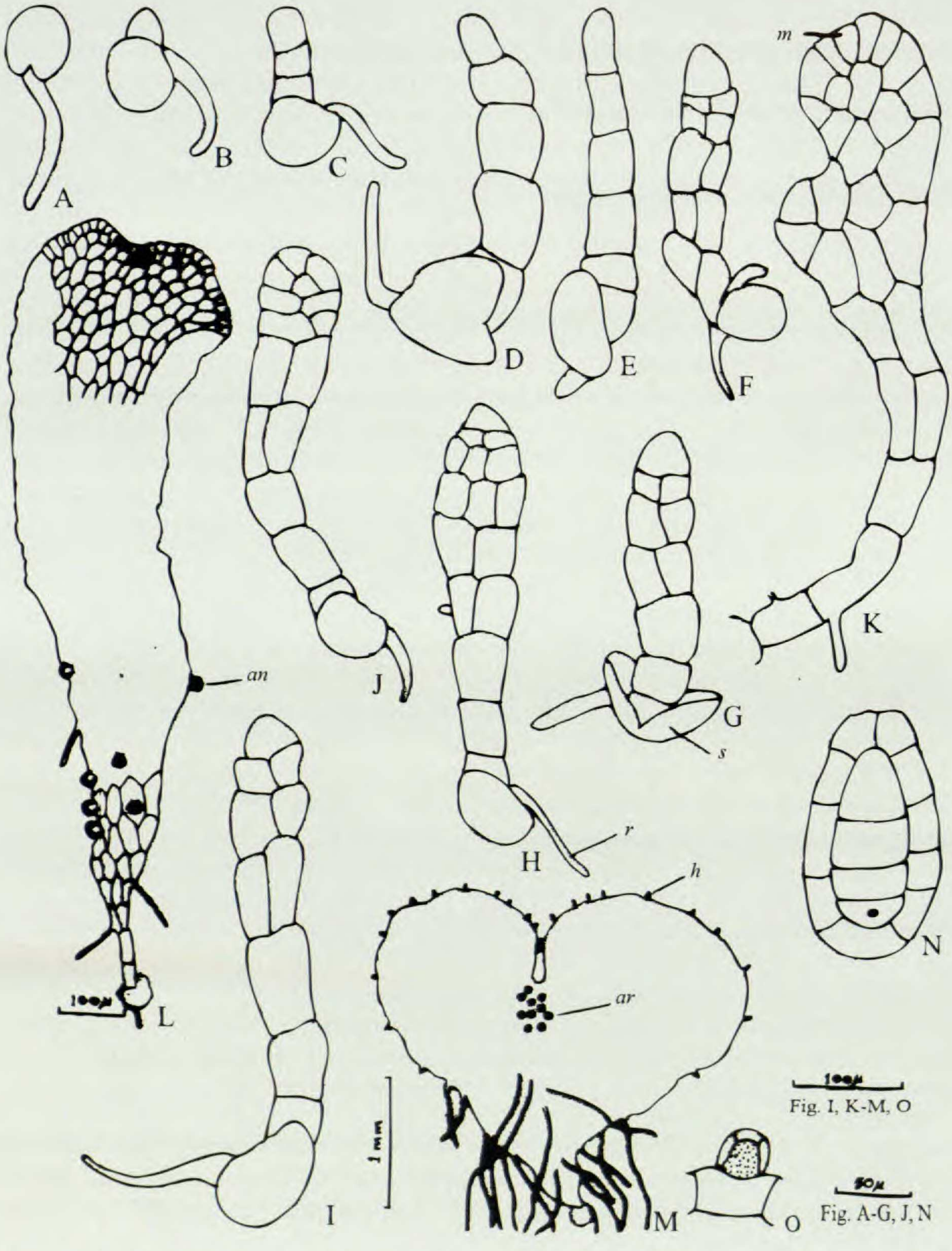

FIG. 1. **A–O.** Different stages of gametophyte development of *Arthromeris himalayensis*. **A.** Germination of spore showing first rhizoidal cell. **B.** Emergence of first prothallial cell. **C–E.** Different stages of filamentous prothallus. **F–J.** Stages in development of non-meristematic prothallial plate. **K.** Establishment of apical meristematic cell. **L.** Development of antheridia on antheridiate prothallus. **M.** Development of archegonia on mature cordate prothallus. **N.** A mature archegonium. **O.** A mature antheridium. **NB**:[*h* = Hair ; *m* = Obconical meristematic cell ; *r* = Rhizoid ; *s* = Spore coat ; *a* = Antheridium ; *ar* = archegonium].

TABLE 1. Time taken for gametophyte development of *Arthromeris himalyensis* (Hook.) Ching.

Sl. No.	Events of the gametophyte development	Total no. of days taken after sowing of spore ± SD
1.	Sowing of spores	0 ± 0
2.	Spore germination	10 ± 1
3.	Formation of mature cordate prothallus	80 ± 3
4.	Initiation of archegonia	112 ± 2
5.	Initiation of antheridia	115 ± 3
6.	Maturation of antheridia	118 ± 3
7.	Initiation of sporophyte	123 ± 2

"*Drynaria* type" development, spore germination results in a slender uniseriate germ filament. A broad spathulate prothallial plate is formed by repeated longitudinal and transverse divisions of its anterior cell and expansion of the resultant daughter cells. The prothallial plate often becomes 5–10 cells or more wide and broadly ovate, but is devoid of any organized meristem. Later, an obconical meristematic cell is differentiated by two oblique divisions on one of the marginal cells at the anterior end of the prothallial

TABLE 2. Classification of gametangial sequence on meristematic prothalli of homosporous ferns (adapted from Verma 1989, 2003).

Sequential bearing of gametangia on meristematic prothalli			
Type	Initial state	Final state	Symbol
A	Antheridiate	Archegoniate.	M → F
	Archegoniate	Persists throughout.	F → F
B	Antheridiate	Antheridia and Archegonia formation.	M → H
	Archegoniate	Antheridia and archegonia formation.	F → H
C	Antheridiate	Antheridia and archegonia formation for some time, then only archegonia formation.	M → H → F F → H → F
	Archegoniate	Same	
D	Antheridiate	Antheridia and archegonia formation for some time, alternating periodicity in the formation of antheridia and archegonia, finally hermaphrodite.	M ↔ H ↔ F ↔ H F ↔ H ↔ M ↔ H
	Archegoniate	Same	
E	Antheridiate	Archegonia formation.	M → F
	Archegoniate	Antheridia and archegonia formation.	F → H
F	Archegoniate	Antheridia and archegonia formation (ephemeral), then antheridia formation.	F → H → M or F → M
G	Archegoniate	Antheridia and archegonia formation simultaneously.	F → H
H*	**Archegoniate**	**Persists throughout.**	**F → F**
	Antheridiate	**Persists throughout.**	**M → M**

Symbols indicate the sequential state of functional sex:
M = Antheridia formation, F = Archegonia formation, H = Hermaphrodite.
Types A, B and C are according to Masuyama (1975 b). Type D, E and F are proposed by Verma (1989). Type G is proposed by Ganguly & Mukhopadhyay (2005). Type **H*** is a new variant type proposed here by current authors.

plate. The young prothallus becomes cordate, the apical meristematic cell is replaced by a pluricellular meristem and a midrib developed. Young prothalli are naked; hairs are usually formed when the prothallial plate becomes cordate (Nayar and Kaur, 1969). The *Drynaria* type of development is characteristic of Cheiropleuriaceae, Dipteridaceae, Gleicheniaceae, Lomariopsidaceae, Loxomaceae, Thelypteridaceae and the majority of the Polypodiaceae genera (Nayar and Kaur, 1969). Smith *et al.* (2006) did not consider Cheiropleuriaceae a separate family; they merged the genus *Cheiropleuria* in the family Dipteridaceae. According to Masuyama's (1975a, b) classification of gametophytes, based on gametangial sequence of development on meristematic prothalli, which was further elaborated upon by Verma (1989, 2003) and Ganguly and Mukhopadhyay (2005), the gametophytes of *Arthromeris himalayensis* resemble 'type A' to some extent. In type A, the archegoniate prothalli persist throughout development but the antheridiate prothalli become archegoniate in the later stages. In *Arthromeris himalayensis*, the sequence of development of the sex organs is different. Here, the archegoniate prothalli remain archegoniate and the antheridiate prothalli remain antheridiate throughout development. Based on the sequence of sex organ development in *Arthromeris himalayensis*, it is identified as a new type, different from the types described by Masuyama (1975a, b), Verma (2003), and Ganguly and Mukhopadhyay (2005). Thus, we propose a new type "Type H" in addition to the existing seven types classified by the previous authors (Table 2).

Gametophyte growth habit can be classified into three basic types in regard to the effect of form on breeding system. Type I is the familiar cordate or butterfly shaped gametophytes of most terrestrial ferns. Type II gametophytes have indeterminate growth and branching and type III gametophytes combine type II growth with production of dispersible gemmae. Type II and type III gametophytes are typical of most epiphytic species (Farrar, 2003). In *Arthromeris himalayensis*, the archegoniate prothalli resemble type I, which is cordate shaped. The antheridiate prothallus was elongated, having indefinite growth. Some of the gametophytes showed clonal elongation. The secondary gametophytes produced antheridia on their margins. Thus, antheridiate prothalli resemble type II gametophytes partially.

The gametophytes of *Arthromeris himalayensis* are long lived (more than 110 days), and the advantage of long-lived gametophytic generation is to promote cross-fertilization (Klekowski, 1973, 1979). Opportunities for gamete exchange between long-lived gametophytes are much higher than for short-lived, non-clonal epiphytic gametophytes.

Most species of Polypodiaceae maintain an antheridiogen system through which the robustly growing female gametophytes induce production of antheridia precociously on the smaller gametophytes growing nearby, thus enhancing the probability of cross-fertilization (Chiou and Farrar, 1997). *Arthromeris himalayensis* may have an antheridiogen system, as antheridia grow on separate prothalli after 3–5 days of initiation of archegonia in cordate

shaped prothalli. This suggests that antheridiogen might have some role in controlling the reproductive system of *A. himalayensis*.

Masuyama (1975b) recognized four basic locations of antheridia on monoecious prothalli: antheridia on the lower part of gametophyte thallus (type L), on the lower half of the wings (LW), on the lower half of the margin (type LM), on the upper half of the central cushion (type UC), or antheridia located all along the margin (type M). As *Arthromeris himalayensis* produces dioecious prothalli, it does not resemble any type as recognized by Masuyama (1975b), though the antheridia located on the lower half of the wings (type LW) as proposed by Masuyama (1975b).

It is interesting to note that the percentage of spore germination is very high; about 79.49 ± 4.66% even after two months of harvesting. This figure indicates that this species produces a high proportion of viable spores, which is likely helpful in the survival of this species. This species is restricted to a certain altitudinal regions (2700–3600 m), thus specific environmental conditions like temperature, annual rainfall, relative humidity (RH), etc. are required for its survival. RH, annual rainfall and altitude have a combined effect on the distribution and reproductive success of this species (Ganguly and Mukhopadhyay, 2008).

Most homosporous fern gametophytes are potentially bisexual and due to continual self-fertilization there is a risk of exposing the lethal genes in homozygous condition. The gametophytic generation has evolved some adaptations to overcome this problem that influence the change of the mating system from intragametophytic to intergametopytic. These adaptations include the gender of the gametophytes, ecology, distribution and duration of gametangia on monoecious prothalli, and longevity of gametophytes and the capacity for vegetative reproduction (Klekowski, 1969; Lloyd, 1974a, b; Masuyama 1975a, b; Soltis and Soltis, 1987). From the above discussions, it may be concluded the *Arthromeris himalayensis* gametophytic generation shows some derived developmental features: 1) dioecious prothalli promotes intergametophytic fertilization (may be of sibling and/or non-sibling mating); 2) archegoniate prothalli that are meristematic and cordate shaped, continuously producing archegonia, increase the chances of sporophyte production; and 3) long-lived gametophytes (more than 110 days) that also promote intergametophytic fertilization.

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